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Polysaccharides of *Aloe vera* induce MMP-3 and TIMP-2 gene expression during the skin wound repair of rat

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ABSTRACT

Polysaccharides are the main macromolecules of *Aloe vera* gel but no data about their effect on extracellular matrix (ECM) elements are available. Here, mannose rich *Aloe vera* polysaccharides (AVP) with molecular weight between 50 and 250 kDa were isolated and characterized. Open cutaneous wounds on the back of 45 rats (control and treated) were daily treated with 25 mg ($n = 15$) and 50 mg ($n = 15$) AVP for 30 days. The levels of MMP-3 and TIMP-2 gene expression were analyzed using real time PCR. The levels of n-acetyl glucosamine (NAGA), n-acetyl galactosamine (NAGLA) and collagen contents were also measured using standard biochemical methods. Faster wound closure was observed at day 15 post wounding in AVP treated animals in comparison with untreated group. At day 10 post wounding, AVP inhibited MMP-3 gene expression, while afterwards MMP-3 gene expression was upregulated. AVP enhanced TIMP-2 gene expression, collagen, NAGLA and NAGA synthesis in relation to untreated wounds. Our results suggest that AVP has positive effects on the regulation of ECM factor synthesis, which open up new perspectives for the wound repair activity of *Aloe vera* polysaccharide at molecular level.

1. Introduction

Extracellular matrix degradation, cell migration, matrix resynthesis and tissue remodeling are the main occurrences during the wound healing [1–4]. Matrix metalloproteinases (MMPs) are a family of zinc dependent proteases that can degrade most components of the extracellular matrix (ECM), activate other proteases and regulate the release of growth factors and cellular receptors [2,3,5]. These proteases activate or inactivate during many normal physiological processes including embryonic development, reproduction, normal wound healing [2,3] and in disease processes such as arthritis [6], tumor metastasis [5] and wound healing failure [7]. Endogenous inhibitors of MMPs, the tissue inhibitors of matrix metalloproteinase (TIMPs), regulate the production and activation of MMPs and play an important role in all aspects of wound repair [1,3,8].

Several studies have demonstrated alterations in MMP-3 and TIMP2 protein levels during wound healing and contraction [1,9–13]. It has been stated that the MMP-3 expression level decreases in patients with impaired wound healing [7] or in mice lacking MMP3 [9,10]. It has been shown that treatment of cutaneous wounds with TIMP2 inhibitors impairs the wound's re-epithelialization and contraction, and decreases the differentiation of cells within the granulation tissue [8,13].

Polysaccharides of plants are essential biomacromolecules that have been used as therapeutic agents for animals and humans for many years. *Aloe vera* is the mucilaginous gel from the parenchymatous cells in the leaf pulp of *Aloe barbadensis*. The main active compounds of *Aloe vera* are polysaccharides [14]. Numerous studies have reported the beneficial effects of *Aloe vera* components on health including antimicrobial, anti-inflammatory, immunomodulatory and antioxidant criteria [14–20].

There is some controversy regarding the beneficial effects of the whole gel extract of *Aloe vera* on wound healing; while many reports support promotion of wound healing by the crude gel extract, other studies did not provide enough evidence to confirm its advantage to the healing processes [15–18]. It has also been reported that treatment with *Aloe vera* gel resulted in delayed wound healing [21]. In another trial, *Aloe vera* gel hindered wound healing in an experimentally induced second-degree burn [22]. The conflicting reports concerning the efficacy of many commercial preparations of *Aloe vera* on wound healing have stimulated research toward the definition of the molecular actions of its main components during wound healing. To date, study of the changes in MMP3 and TIMP2 gene expression in wounds treated by AVP remains incomplete. The purpose of the present study was to investigate the effect of topical application of AVP on MMP3 and TIMP2 gene expression levels and also hexosamines and collagen synthesis during cutaneous wound healing in rat.

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2. Experimental

2.1. Preparation of Aloe vera polysaccharides

Aloe vera polysaccharides (AVP) were prepared according to the method described by Wu et al. [23]. Full size mature leaves were cut from the *Aloe vera* cultivated in Shiraz, Iran and the rind removed. The colorless parenchyma was ground in a blender and heated in distilled water at 70–80 °C for 2 h. The filtrate of the obtained extract was condensed in evaporator and mixed with cold 95% ethanol. The sample was stood overnight at 4 °C and polysaccharide precipitates were collected from the alcoholic phase by centrifugation at 12,000 × *g* and repeatedly washed sequentially with ethanol, acetone and ether, respectively. AVP was redissolved in distilled water and deproteinized by treatment with trichloroacetic acid as described previously [24]. Supernatant containing AVP was obtained after centrifugation at 12,000 × *g* for 10 min and it was then precipitated with a 3-fold volume of 95% ethanol. AVP was sterilized using 0.45 μm filter, lyophilized and stored at room temperature until use. Bacterial test of lyophilized AVP was performed using tryptic soy agar and broth medium. Endotoxin concentration of prepared AVP was tested using Toxin Sensor™ Chromogenic LAL Endotoxin Assay Kit (Genscript, USA) and free endotoxin AVP was used for the subsequent experiments. Before use, AVP was prepared as gel by dissolving 25 mg or 50 mg of lyophilized powder in 0.5 ml distilled water.

2.2. Carbohydrate composition analysis

The isolated APS was subsequently subjected to a Sephacryl S-400 gel filtration column chromatography (3.5 cm × 100 cm) (Sigma, Germany), eluting with the deionized water at a flow rate of 25 ml/h. The elute was collected as 3-ml fractions. Phenol sulfuric acid method was used to measure the carbohydrate content in each fraction [25]. To determine the molecular weight of APS fractions over a Sephacryl S-400 gel column chromatography (3.5 × 100), a set of Dextrans (270, 150, 80, 50, 12 kDa) (Pharmacosmos, Denmark) was used as references.

HPLC analysis was used to determine the free sugars in the isolated AVP. Twenty mg of AVP was hydrolyzed using 2 ml of 0.5 M sulphuric acid for 20 h in a boiling water bath as described previously [26]. The analysis was performed on operating system model Shimadzu (SCL-10AVP) equipped with RI detector RID-10A and high pressure pump LC-10ADVP. Separation and determination were performed on Shodex column sugar SC1011 using distilled water as mobile phase with 1 ml/min flow rate.

2.3. Experimental animals

Forty five male adult Wistar rats weighing 200–300 g were housed in a temperature-controlled room (23 ± 1 °C) with a 12 h light/dark cycle and were provided rat chow (Pars, Tehran, Iran) and water *ad libitum*. Rats were housed one per cage. All the animals were cared for according to the guide for the care and use of laboratory animals by the National Academy of Sciences (National Institutes of Health publication No. 86-23). The rats were allowed to acclimatize for one week before the beginning of the experiment.

2.4. Wound creation

The backs of the animals were shaved and sterilized with 70% EtOH. The animals were anesthetized by intramuscular injection of 2 mg/kg xylazin HCl (Xylasin 2%®, Alfasan, Woerden, Netherlands) as premedication and 60 mg/kg ketamine HCl (Ketamin 10%®, Alfasan, Woerden, Netherlands) for anesthesia. A circular full-thickness 1 cm in diameter skin wound was then aseptically created on the back of each rat.

2.5. Grouping design and therapeutic regimens

The animals were divided into three groups of fifteen animals each including: (A) Control Group with wound and no treatment, (B) Test Group I with wound and daily treatment with 25 mg AVP in 0.5 ml distilled water, (C) Test Group II with wound and daily treatment with 50 mg AVP in 0.5 ml distilled water.

The rats were topically treated once daily for 30 days. The animals in each group were evaluated daily, but euthanized on the 10th, 20th and 30th day after wound creation and the entire dorsal skin encompassing healing wounds was excised and stored at −70 °C until gene expression and biochemical analyses.

2.6. Gross morphology of the wounds

The wound area was observed and photographed daily. The photographs were transferred to Scion image software for morphometric analysis including measurement of the large and small diameter of the wounds and calculation of area of the wounds [27]. Rate of wound closure was calculated by the following equation:

$$\text{Wound area (\%)} = \frac{\text{wound area at day } X}{\text{wound area at day } 0} \times 100.$$

Percent of wound closure was calculated by the following formula: Percent of wound closure on day $X = 100 -$ wound area percent on day X [27].

2.7. Biochemical analyses

Estimation of hydroxyproline and collagen concentrations was done as described previously [4]. The excised granulation tissues were weighed, defatted in chloroform:methanol mixture (2:1, v:v), hydrolysed in 6.0 N HCl for 18 h at 110 °C and evaporated to dryness. The hydrolysate was neutralized to pH 7.0 and subjected to chloramine-T oxidation; all the test tubes were placed in a water bath at 60 °C for 20 min. The reaction was terminated by addition of 1 ml (0.4 M) perchloric acid, the color was developed by adding 1 ml paradimethyl aminobenzaldehyde (PDAB) and was read spectrophotometrically at 557 nm. The amount of hydroxyproline in each sample was measured using the regression curve from the hydroxyproline standards (0.0, 0.5, 1.0, 2.0, 4.0 and 6.0 mg hydroxyproline) and reported as mg/100 mg tissue. The collagen content was estimated from the hydroxyproline concentration of tissue through multiplication by the factor 7.46 [4].

n-Acetyl glucosamine (NAGLA) and n-acetyl galactosamine (NAGA) concentrations of dry granulation tissues were measured as described previously [4]. Briefly, alkaline hydrolysate was converted into pyrrole derivatives by acetylacetone and was subsequently treated with PDAB to a red-colored solution which was measured at 585 nm by spectrophotometry.

2.8. RNA isolation and reverse transcription

Total RNA was extracted from frozen granulation tissues using RNX-Plus reagent according to the manufacturer's procedure (Cinnagen Inc., Tehran, Iran), dissolved in dimethyl pyrocarbonate treated water and quantified at a wavelength of 260 nm by nanodrop spectrophotometry (Eppendorf, Hamburg, Germany). The integrity of RNA was verified by optical density (OD) absorption ratio OD260 nm/OD280 nm between 1.8 and 2.0. For genomic DNA removal an in-solution DNase digestion was carried out by treating 1 μg of RNA with 2 units of DNase I (Fermentas Inc., Vilnius, Lithuania).

First-strand cDNA was synthesized from 1 μg of RNA using RocketScript RT PreMix kit (Bioneer Corporation, South Korea) and Oligo dT following the manufacturer's instructions.

2.9. Quantitative real time PCR

A quantitative real-time PCR using qPCR™ Green Master Kit for SYBR Green I® (Jena Biosciense, Germany) was developed for detecting relative MMP3 and TIMP2 mRNA levels in rat skin, in an ABI 7500 real-time PCR detection system (ABI, USA). Relative expression levels of MMP3, TIMP2 transcript were normalized to RNA loading for each sample using GAPDH mRNA. Specific sets of primers (Macrogen, Seoul, South Korea) used for amplification of all genes were designed using Beacon Designer 7.1. Sequences of sense and antisense primers (Macrogen, Seoul, South Korea) for the MMP-3, TIMP-2 and GAPDH were as follows: for MMP-3: 5′-cacacctgactcggttccgcc-3′ and 5′-gttggctggcgtcccaggtt-3′, for TIMP-2: 5′-gctggacgttggaggaaaga-3′ and 5′-tgatgctaagcgtgtcccag-3′ for GAPDH: 5′-agttcaacggcacagtcaag-3′ and 5′-tactcagcaccagcatcacc-3′.

Real time PCRs were performed using qPCR™ Green Master Kit in a final volume of 20 μL containing 3 μL DNAse treated cDNA. The reactions were performed with the following settings: 5 min of pre-incubation at 95 °C followed by 40 cycles for 15 s at 95 °C and 45 s at 60 °C. Reactions were performed in triplicate. A reaction without cDNA was performed in parallel as negative control.

Relative quantification was performed according to the comparative $2^{-\Delta\Delta Ct}$ method as described previously. The result for the gene expression was given by a unitless value through the formula $2^{-\Delta\Delta Ct}$. For analysis of qRT-PCR results based on ΔΔCt method StepOne™ software was used. Validation of assay was performed to check that the primer for the GAPDH, MMP3 and TIMP2 had similar amplification efficiencies [28].

2.10. Statistical analyses

Data analyses were done using the SPSS 18.0 software package (SPSS Inc., Chicago, IL, USA). One-way ANOVA was used to test differences between various means, followed by *post hoc* Tukey test. All experimental data were presented as the mean ± standard deviation (SD). The level of significance for all tests was set at $P < 0.05$.

3. Results

Isolated AVP displayed no absorbance at 280 nm and was revealed to be free of any protein. AVP was fractionated into three distinct peaks (AVP1-3) by Sephacryl S-400 gel filtration chromatography (Fig. 1A). The yields of the fractions, calculated according to Dubois method, were about 55%, 20% and 25%, respectively. According to Dextran standards, the molecular weights of AVP1, AVP2 and AVP3 were about 250 kDa, 150 kDa and 50 kDa, respectively. Quantitative HPLC analysis of AVP fractions revealed that AVP1 and AVP2 shared similar monosaccharide composition and had mannose, galactose, arabinose and glucose in the ratio of 88%, 6%, 4% and 2%, respectively. AVP3 had 57% mannose, 30% galactose, 8% arabinose and 5% glucose (Fig. 2).

As shown in Fig. 3A–D, treatment of wounds with 25 mg and 50 mg AVP resulted in higher rate of wound closure at 15 days post wounding. Treatment with 50 mg AVP showed faster closing rate. The wounds that were topically treated by 25 and 50 mg of AVP were closed and re-epithelized on days 21 and 18 post wounding respectively, as compared to the control groups where the injured area was healed at 27 days post wounding (Fig. 3A–D).

Fig. 4A represents the expression levels of MMP3 gene in granulation tissues of the treated and untreated rats. The MMP-3 mRNA content of the granulation tissues reached maximum levels 30 days after wound creation in the control and treated groups ($P < 0.05$, Fig. 4A). MMP-3 displayed different expression pattern in response to AVP during earlier and later stages of healing. At the 10th day post wounding, the animals that had been treated by both doses of AVP displayed significant inhibition in the expression level of MMP-3 gene compared to the untreated animals ($P < 0.05$, Fig. 4A). Increase in the concentration of AVP resulted in a progressive decrease in the mRNA level of MMP-3 ten days after wound creation ($P < 0.05$, Fig. 4A).

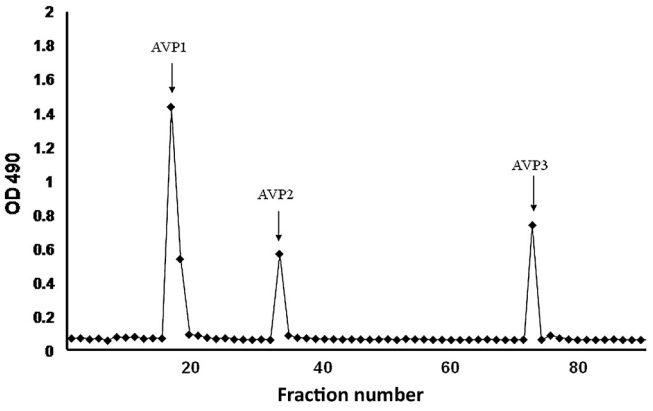

Fig. 1. Characterization of fractionated polysaccharides from *Aloe vera* gel. The resulting polysaccharide fractions (AVP1–AVP3) from Sephacryl S-400 column eluted with deionized water at a flow rate of 25 ml/h. Using dextrans as standard the molecular weight of AVP1, AVP2 and AVP3 were determined to be about 250 kDa, 150 kDa and 50 kDa, respectively.

On days 20 and 30 post treatment, however, MMP-3 mRNA levels were significantly higher in both treated groups when compared to the untreated control wounds ($P < 0.05$, Fig. 4A). There was no significant difference in the MMP-3 mRNA level between the rats that received 25 or 50 mg of AVP after 20 and 30 days of treatment ($P > 0.05$, Fig. 4A)

As shown in Fig. 4B, the mRNA level of TIMP-2 reached its maximum level at day 20 after wound creation in all groups ($P < 0.05$, Fig. 4B). In the treated and untreated rats, however, the TIM-2 levels returned to their levels at day 10 post wounding ($P < 0.05$, Fig. 4B). At all post wounding days, the AVP treated animals expressed greater levels of TIMP-2 mRNA than did the untreated rats ($P < 0.05$, Fig. 4B).

Fig. 5A and B illustrates the effect of AVP on the NAGLA and NAGAA levels in the rats of the experimental groups. As shown in Fig. 5A and B, the NAGLA and NAGAA levels reached their maximum level at day 20 after wound creation in all groups ($P < 0.05$). Their levels decreased after day 20 and returned to their levels at day 10 in all experimental groups ($P < 0.05$, Fig. 5A and B). Topical application of AVP at doses of 25 and 50 mg resulted in significantly higher ($P < 0.05$) levels of NAGLA and NAGAA compared to those of the untreated rats on the 10th, 20th and 30th day after treatment ($P < 0.05$, Fig. 5A and B). It was observed that the rats in Group 2 had higher NAGLA and NAGAA contents when compared to the animals of Group1 throughout the post-treatment periods ($P < 0.05$, Fig. 5A and B).

The collagen contents of the granulation tissues of the animals of all three groups are presented in Fig. 6. Maximum concentration of collagen in the injured area of the animals in all groups was observed at day 20 post wounding ($P < 0.05$, Fig. 6). Both concentrations of AVP resulted in a significantly higher level of collagen content compared with control group at all post-wounding intervals ($P < 0.05$, Fig. 6). The collagen concentration then gradually started to decrease from day 20 and returned to its level at day 10 in all experimental groups ($P < 0.05$, Fig. 6). However, the wounds in the animals of Group 2, which were treated by 50 mg of AVP

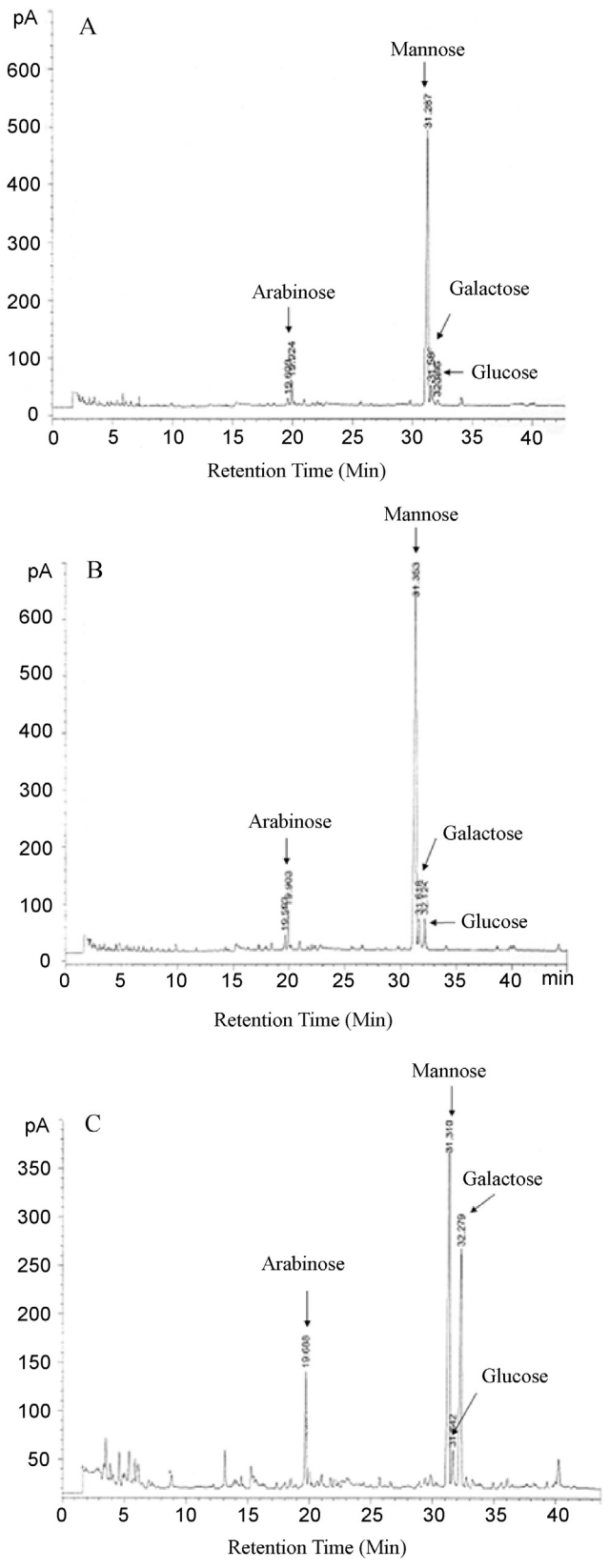

Fig. 2. Sugar content of fractionated *Aloe vera* polysaccharides, AVP1 (A), AVP2 (B) and AVP 3(C), which were analyzed using HPLC. AVP1 and AVP2 shared similar monosaccharide composition and had mannose, galactose, arabinose and glucose in the ratio of 88%, 6%, 4% and 2% respectively. AVP3 had 57% mannose, 30% galactose, 8% arabinose and 5% glucose.

had higher collagen content when compared to those of Group1 animals throughout the treatment ($P < 0.05$, Fig. 6).

4. Discussion

Numerous data from different studies have shown extracellular matrices including MMPs and TIMPs gene expression changes during the wound healing. The key regulators of ECM turnover are MMP-3 and its endogenous inhibitor TIMP-2 [2,3,5]. Regulatory effects of topical application of *Aloe vera* gel on the gene expression of MMP-3 and TIMP-2 have not been reported so far.

Based on our findings, maximum concentration of MMP-3 mRNA was observed at day 30 after wound creation. Exposure of wounds with AVP resulted in marked upregulation of MMP-3 genes in granulation tissue at this time. Moreover, treatment of wounds with AVP increased the maximum levels of MMP-3 gene expression at the 20th and 30th day post treatment compared to the untreated wounds. Upregulation of MMP-3 by AVP during the maturation phase of the wound healing was paralleled by the faster closure of the treated wounds. The wounded skins were closed as early as 18 and 21 days after treatment with 25 and 50 mg of AVP, respectively.

MMP-3 is secreted by fibroblasts and its activity is particularly important in regulating wound healing progression [4,9,10,29]. MMP-3 has a broad range of ECM substrates, can activate pro-MMPs and can release cell or matrix-associated growth factors [30–32]. Although MMP-3 function is required in all stages of wound healing processes, its activity is much more important during the final stages of wound healing, including resolution and contraction phases of wound healing [1,2,10,29]. Reduction in MMP3 content or addition of its synthetic inhibitor results in slowed wound contraction and closure [9,10,13,29]. It has recently been reported that stimulation of MMP-3 gene expression by silibinin during the wound repair results in faster closure of dermal wound [4]. Based on the above findings we hypothesized that early closure of the wounded skin in those animals treated with AVP may be directly related to the high level of MMP-3 gene expression in comparison to the untreated animals. Down regulation of MMP-3 gene has been reported in wounds that exhibited impaired contraction and closure relative to normal healing [7,9,10]. From these data, we concluded that AVP might have some ingredients which upregulate MMP-3 gene expression in a dose dependent manner during the final stage of wound repair.

Although AVP had stimulatory effect on MMP-3 mRNA production during the final stage of wound healing, it downregulated MMP-3 gene expression at the early stage of wound repair. Increase in concentration of *Aloe vera* resulted in progressive decrease in the mRNA level of MMP-3 at this time. Some contradictory findings on the effect of *Aloe vera* on wound healing have previously been reported in experimental animals or human. Schmidt and Greenspoon [21] reported that treatment of dermal wound by *Aloe vera* gel resulted in a significant delay in wound repair during the first week of healing compared to those of the normal unassisted healing. Other researchers have reported similar findings during wound healing in second-degree burns [22]. The mechanism by which MMP-3 is differentially expressed in AVP treated wounds remains unknown. We concluded that downregulation of MMP-3 at the first stage of dermal wound repair may delay wound healing, which supports some of the previous findings, which indicate the inhibitory effect of *Aloe vera* in the early stage of dermal wound healing.

According to our findings, maximum amount of TIMP-2 mRNA was detected on day 20 post wounding in all groups and it then tended to decrease afterward. Expression of TIMP-2 was potently upregulated by both doses of AVP after 20 and 30 days of treatment compared to those of the untreated animals. Wound contraction and closure, an important component of the resolution phase

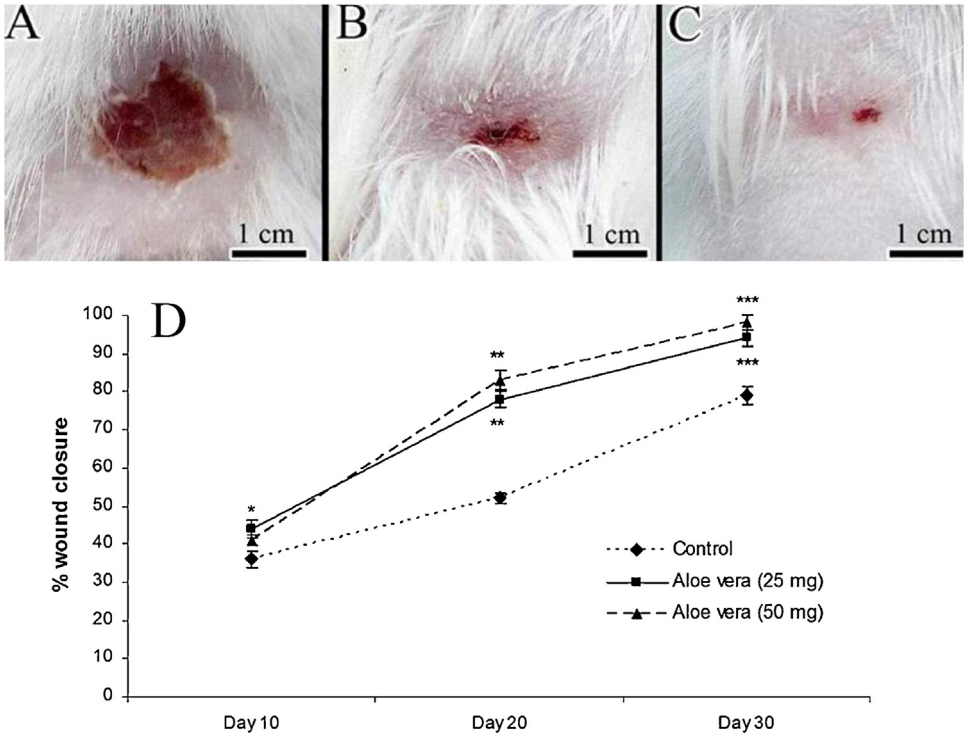

Fig. 3. Effect of topical application of AVP on wound healing at 15 days post wounding. (A) Control, untreated group. (B) Wound treated with 25 mg *Aloe vera* gel. (C) Wound treated with 50 mg *Aloe vera* gel. (D) Rate of wound closure of AVP treated and untreated wounds. Data are given as mean ± S.D. for five animals in each group. Statistically significant results are indicated as * at $P < 0.05$.

of wound healing, is susceptible to regulation by inhibitors of metalloproteinases [1,2]. Treatment of cutaneous wounds with GM6001, a synthetic inhibitor of TIMPs, leads to impaired re-epithelialization but it also decreases wound contraction and alters the differentiation of cells within the granulation tissue [13]. It has also been stated that the cell migration potential is elevated in *in vitro* situation in the presence of metalloproteinase inhibitors and this may stimulate early wound closure [1,11]. TIMP2

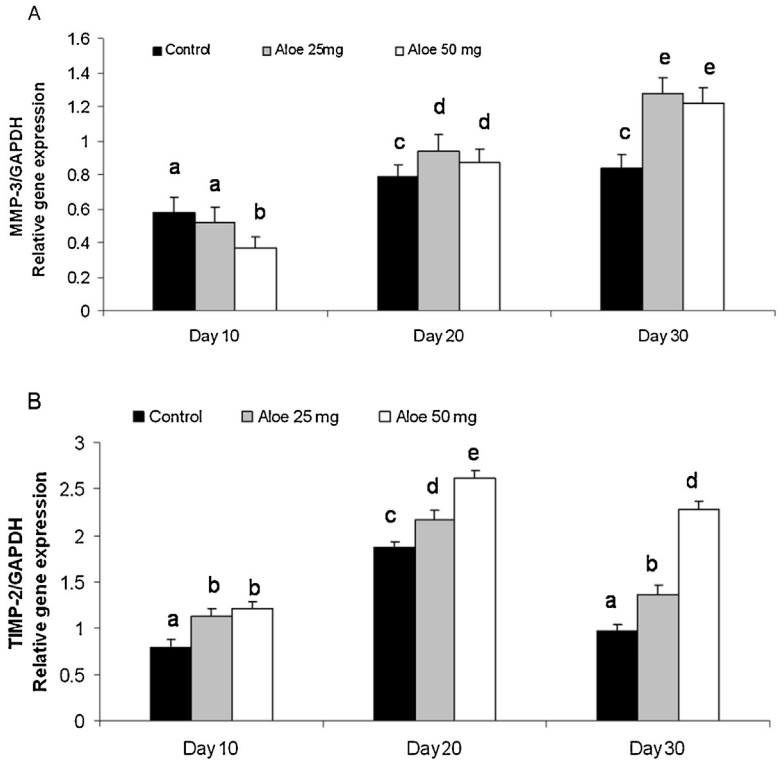

Fig. 4. Effect of topical application of different doses of AVP on MMP-3 (A) and TIMP-2 gene expression at the 10th, 20th and 30th day after wound creation. Different letters above each bar represent significant difference at $P < 0.05$.

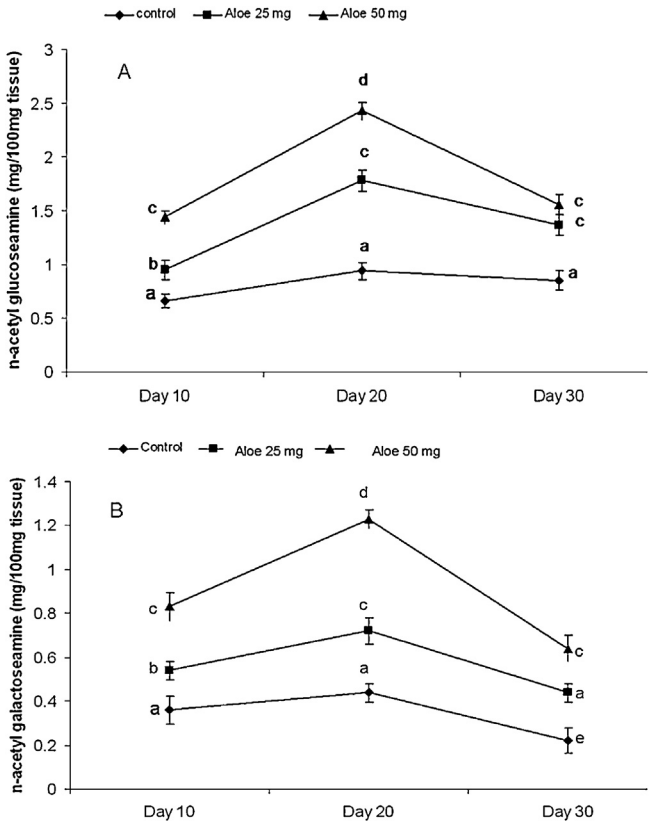

Fig. 5. Effect of topical application of different doses of AVP on n-acetyl glucosamine (A) and n-acetyl galactosamine (B) concentrations at the 10th, 20th and 30th day after wound creation. Different letters above each bar represent significant difference at $P < 0.05$.

accelerates keratinocyte migration in culture and *in vivo* [12]. Low MMP-3 gene expression, consistent with the high level of TIMP-2 mRNA observed in the final stage of dermal wound repair in this study, indicates the role of MMP/TIMP balance in acceleration of the connective tissue remodeling, cell migration and wound closure.

It was also concluded that regulation of MMP-3 and TIMP-2 genes by AVP might have a positive effect on the levels of required substances such as glycosaminoglycans and collagen for wound proliferation and extracellular matrix remodeling. To support this hypothesis, changes in MMP-3 and TIMP-2 gene expression during the wound healing were found to be in a similar state due to changes in the collagen and hexosamines concentrations. The collagen content of the treated wounds with the AVP was found to be higher than in the untreated animals and reached its maximum level by 20 days after treatment. It has been shown that AVP components stimulate collagen synthesis, change its composition and inhibit collagenase activity during the wound repair [15,18]. From these results, it may be concluded that AVP inhibits MMP-3 and induces TIMP-2 genes expression during the early stage of dermal repair, resulting in decreased collagen breakdown and preservation of higher amounts of collagen content in the injured area. In the final stage of dermal repair, by enhancing the TIMP-2 gene expression and reducing the MMP-3 mRNA level, AVP results in declined collagen synthesis.

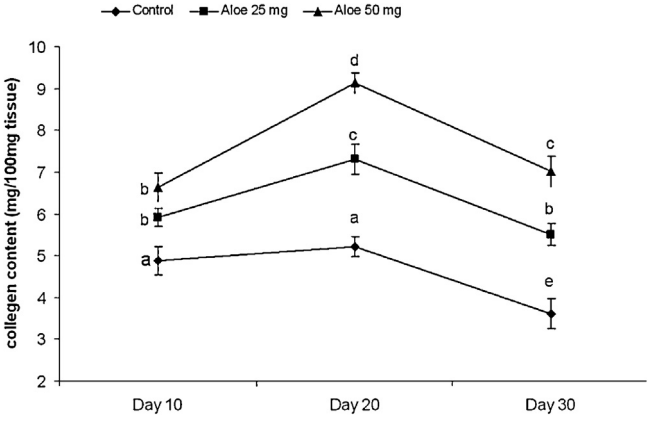

Fig. 6. Effect of topical application of different doses of AVP on collagen concentration at the 10th, 20th and 30th day after wound creation. Different letters above each bar represent significant difference at $P < 0.05$.

It was also demonstrated here that treatment of the wounded skin with AVP led to induction of glycosaminoglycans production [16]. NAGLA and NAGAA are the key substrates for synthesis of hyaluronic acid and dermatan sulfate. These GAGs play an important role in the cellular proliferation and the orderly deposition of structural matrix during wound repair [33] and some reports have shown that *Aloe vera* stimulates the production of these components during dermal wound repair [16].

A number of natural compounds isolated from plants have been used as wound healing modulators in animals and human [4,18,34]. However, many contradictory findings about the efficacy and safety of these compounds to treat dermal wounds have been found [35]. Our results open up new perspectives for the wound healing activity of AVP at molecular level.

The results presented here demonstrated that AVP, at transcriptional level, regulate MMP-3 and TIMP-2 gene expression during the dermal wound repair and it may influence the granulation tissue formation and wound closure by increased production of extracellular matrix constituents including glycosaminoglycans and collagen. Our results also suggest that AVP has different effects on the production of important ECM remodeling factors including MMP-3, TIMP-2, collagen and hexosamines, and its controversial effects on wound healing may be due to these alternative actions.

Conflict of interest

The authors declare that they have no conflict of interest.

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